Reproductive Behavior and Underwater Calls in the Emei Mustache Toad, *Leptobrachium boringii*

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Abstract   The Emei mustache toad, *Leptobrachium boringii* (Megophryidae), is terrestrial but breeds in permanent streams. Primarily, it has a polygynous mating system and polyandry also occurs. The species vigorously defends submerged nest sites. We report on a reproductive study at Mt. Emei, western China conducted in 2004, 2006, 2007, 2009, and 2011 during the breeding season. Males produce both advertisement and courtship calls underwater, and the latter differs from the former in having an additional trill-like last note of low intensity. Females might determine the oviposition location and hence the egg mass location. A low, submissive posture can be adopted by the male to avoid being attacked by other males. When fighting, the maxillary nuptial spines are employed as weapons, and males hold their opponents on their spines with the forelimbs that enlarge during the breeding season. Male body size and the quantity of fight wounds on its ventral side are positively correlated. Satellite male mating might exist in *L. boringii*. Additionally, underwater calls from another population of this species and a population of *L. leishanense* generally are similar to those of the population from Mt. Emei.

Keywords   advertisement call, courtship call, underwater call, male combat, male weaponry, nest inspection, satellite male, reproductive tactic

1. Introduction

In the Asian frog genus *Leptobrachium* (Megophryidae), males of four closely related species, *L. boringii*, *L. leishanense*, *L. ailaonicum*, and *L. liui*, exhibit several unusual traits for anurans. These features include calling underwater and having strong maxillary nuptial spines, conspicuously enlarged arms, and loose skin during the breeding season (Ye *et al.*, 1993; Wells, 2007; Rao and Wilkinson, 2008; Brown *et al.*, 2009; Matsui *et al.*,

2010; Zheng *et al.*, 2010). Sound travels much faster in water than in air, and a sound generated at a given energy produces a much higher sound pressure in water than in air (Elepfandt, 1996). However, in shallow water, a typical habitat of frogs, the transmission of sound is affected by high attenuation of low frequencies and by reflection from both the water surface and the bottom substrate (Rogers and Cox, 1988; Forrest *et al.*, 1993). Probably all anurans are capable of underwater hearing, yet underwater calling is known to occur only in the Pipidae, Ceratophryidae, Ranidae, and possibly the Bombinatoridae (Given, 2005; Wells, 2007; Frost, 2011). As an exception to the basic mechanism of sound production in frogs, pipids produce calls when the arytenoid cartilages are suddenly pulled apart (Yager, 1992). Despite the absence of sonograms,

all of the four species of *Leptobrachium* mentioned above are reported to be capable of vocalizing underwater (Wu and Yang, 1981; Gu *et al.*, 1986; Dubois and Ohler, 1998; Ho *et al.*, 1999). In many anurans with spines or tusks, such structures are used as weapons in male-male combat (Shine, 1979; Wells, 2007). The nuptial spines of these species of *Leptobrachium* are presumed to be employed as weapons in male combat (Dubois and Ohler, 1998; Rao and Wilkinson, 2008; Zheng *et al.*, 2008). In *L. boringii*, underwater male combat using spines is documented by other researchers (Hudson *et al.*, 2011) and by us in 2011. Hypertrophied forelimbs are also associated with male combat, such as wrestling, in many species (Wells, 2007). Males of a few species are known to use spines on the front feet to jab opponents in fights (e.g., Rivero and Esteves, 1969; Wells, 1977), suggesting that the increased arm strength may also enhance the power of the weapon. Details of the fighting behavior of *L. boringii* can be used to explore such a possibility. Both calling and fighting are highly energy-expensive (Ryan, 1988; Wells, 2001) and these activities may be associated with the seasonal appearance of loose skin, which may aid in aquatic cutaneous respiration (Ho *et al.*, 1999; Rao and Wilkinson, 2008).

The terrestrial mustache toad *L. boringii* breeds in permanent streams (Liu and Hu, 1961). Its distribution is restricted to several mountain regions of western China, in valleys covered by broad-leaved forest at elevations between 600 m and 1700 m above sea level. On Mt. Emei, its breeding season begins in the latter half of February and lasts through March. At the onset of the breeding season, males undergo a remarkable transformation. They usually develop 10 to 16 keratinized spines on their upper jaws, the arms become much thicker, and the skin on the dorsum, flanks, and thighs becomes loose. Males migrate to their breeding stream, construct submerged nests under flat rocks, and call in the nest during the night (Wu and Yang, 1981; Zheng *et al.*, 2010). The roof rock is often larger than 0.1 m^2. Pre-oviposition behaviors including physical contact of the male by the female occur. At the start of the oviposition process, the male shifts from an inguinal amplexus position forward and toward one flank of the female. Mates keep on engaging in such an asymmetrical amplexus during oviposition and produce a doughnut-shaped egg mass (Zheng and Fu, 2007). Attached to the undersurface of the rock, egg masses range in size from 35 to 70 mm in diameter. The number of eggs in each clutch ranges from 189 to 346, and multiple egg masses often are found in one nest (Figure 1) (Wu and Yang, 1981; Fei and Ye, 1984; Ye *et al.*, 1993). Most egg masses are deposited early in the breeding season. A female probably produces one clutch of eggs in a breeding season and usually leaves the stream after egg deposition. In contrast, with little or no additional mating opportunity, most mated males remain in their nests for a considerable period of time (e.g., > 25 days), suggesting that they provide paternal care to the offspring (Fei and Ye, 1984; Ye *et al.*, 1993; Zheng *et al.*, 2010). However, the resident male is not necessarily the father of all the eggs in its nest or in an egg mass (Zheng *et al.*, 2010). This implies the existence of alternative male mating tactics, which are common in anurans and other vertebrates (Andersson, 1994; Wells, 2007). Newly hatched tadpoles are found in late March and early April. The tadpoles may take three years to reach metamorphosis and their total length can exceed 100 mm (Liu and Hu, 1961; Wu and Yang, 1981).

Figure 1 Six egg masses of *Leptobrachium boringii* on the bottom surface of the roof rock of a nest. Numbers indicate the position of each egg mass.

We have accumulated data on the reproductive biology of *L. boringii* over the past few years. In this paper, we report characteristics of two types of underwater calls, describe female nest inspection and male subdominant and combat behaviors, present a relationship between male body size and the amount of fight wounds, and describe select spatial and temporal aspects of male behavior. These data suggest the occurrence of two alternative male reproductive tactics.

2. Materials and Methods

We surveyed three study sites located in montane regions of western China. They were small, rocky, permanent streams, each 1–2 m wide, covered by broad-leaved

forest. Fieldwork on *L. boringii* was mainly conducted at Mt. Emei (Site Emei; N 29°34', E 103°25', elevation 650–900 m, about 1.5 km in length) over five years. In 2004 (from 3 March to 24 April), 2006 (28 February to 29 March), and 2007 (28 February to 2 April), we observed the middle and latter parts of the breeding season and recorded male calls. In 2009 (16 to 21 February) and 2011 (23 to 28 February), fieldwork was carried out in the early part of the season. In the other two sites, Mt. Badagong (Site Badagong; *L. boringii*; N 29°47', E 110°06', elevation 1400 m; 23 March 2006) and Mt. Leigong (Site Leigong; *L. leishanense*; N 26°24', E 108°16', elevation 1060 m; 20 to 22 November 2004), male calls were recorded during the breeding season.

Frogs were located mostly by turning rocks and in some cases by their calls. When a frog was found, it was weighed to the nearest 0.5 g using an Avinet spring balance, its snout-vent length (SVL) was measured to the nearest 0.1 mm with calipers, and it was checked for potential wounds caused by possible aggressive interactions between males (Dubois and Ohler, 1998). In 2004, 2006, and 2007, two to three toes were clipped for individual marking. In 2009, markings on top of the head (Figure 2) were photographed and used for identifying individuals (Ferner, 2010). The number of egg masses in the nest was counted, and the locations of egg masses were noted when feasible. The frog was then released to its nest, which had been carefully restored and marked with a unique number. After the initial survey, several follow-up checks were made to monitor the frogs and nests. If a male had left its nest, possible nest sites in the vicinity were checked.

Two artificial nests were used for behavioral observations and recording calls. Each nest was placed close to the breeding stream. At Site Emei, the nest was carefully constructed in an aquarium to simulate a natural one (see Zheng and Fu, 2007, for details). A low level of illumination was provided by a red light at night. In 2006, the behaviors of two males and one gravid female in it were observed, and the calls of one male were recorded. Eggs were clearly visible through the abdominal wall of the female. At Site Leigong, the other artificial nest was constructed in a plastic container (diameter 40 cm, depth 45 cm) with water and rocks from a breeding stream. In 2004, two males and two females were kept together in it, and calls were recorded during the night. Dissection determined that both females had laid all of their eggs.

Male calls were recorded with a Marantz PMD 201 cassette recorder and an Aquarian Audio Products H1 hydrophone. Calls were recorded at a distance of approximately 0.3–0.6 m in the field or 0.1–0.2 m when an artificial nest was used. Water temperature near the natural nest was recorded at the same time to the nearest 1°C with a digital thermometer. Recorded calls were digitized at 44.1 kHz and 16 bits using Adobe Audition version 3.0 (Adobe Systems Inc.) and analyzed using Praat version 5.2.26 (Boersma and Weenink, 2011). Call length, call rate, note duration, note repetition rate, pulse rate, and dominant frequency were measured following the methods of Cocroft and Ryan (1995). The fundamental frequency was determined, following Duellman (1970), by measuring the distance in Hz between several successive adjacent harmonics and dividing the total measurement by the number of harmonics measured. Calls were categorized into different types, e.g., the advertisement call, following Wells and Schwartz (2007).

Statistical analyses were performed with SPSS version 12.0. All measurements were first tested for normality using Kolmogorov-Smirnov test. A paired *t*-test was used to examine whether there were differences between notes within a call. A correlation between male body size and the ranked amount of wounds on its body was tested with two-tailed Spearman correlation. The likelihood ratio chi-square analysis was performed to assess the relationship between altitude and tendency of staying in the nest for males, in the early part of a breeding season. A sequential Bonferroni correction (Holm, 1979; Rice, 1989) was applied for multiple tests.

3. Results

3.1 Male calls of *L. boringii* Two types of calls were recorded from submerged nests, advertisement and courtship calls. The most common advertisement call contained one note. Two-note calls often were recorded in choruses or when a female was present (Figure 3). Three-note calls (quite rare), a four-note call, and a five-note call were recorded only in choruses or when a female was present. At Site Emei, courtship calls of two males, male #0014 in the artificial nest and male #1001 in a natural nest, were recorded when a female was present. This type of call contained two to four notes. We did not observe any individuals giving calls out of the water.

The note of the advertisement call was composed of many clear pulses, which were repeated at a near constant rate across about the first 3/4 of the note duration. The remaining pulses (usually three or four) were repeated at gradually increasing intervals. The highest intensity was observed in the second half of the note. The note also was characterized by slight upward frequency modulation
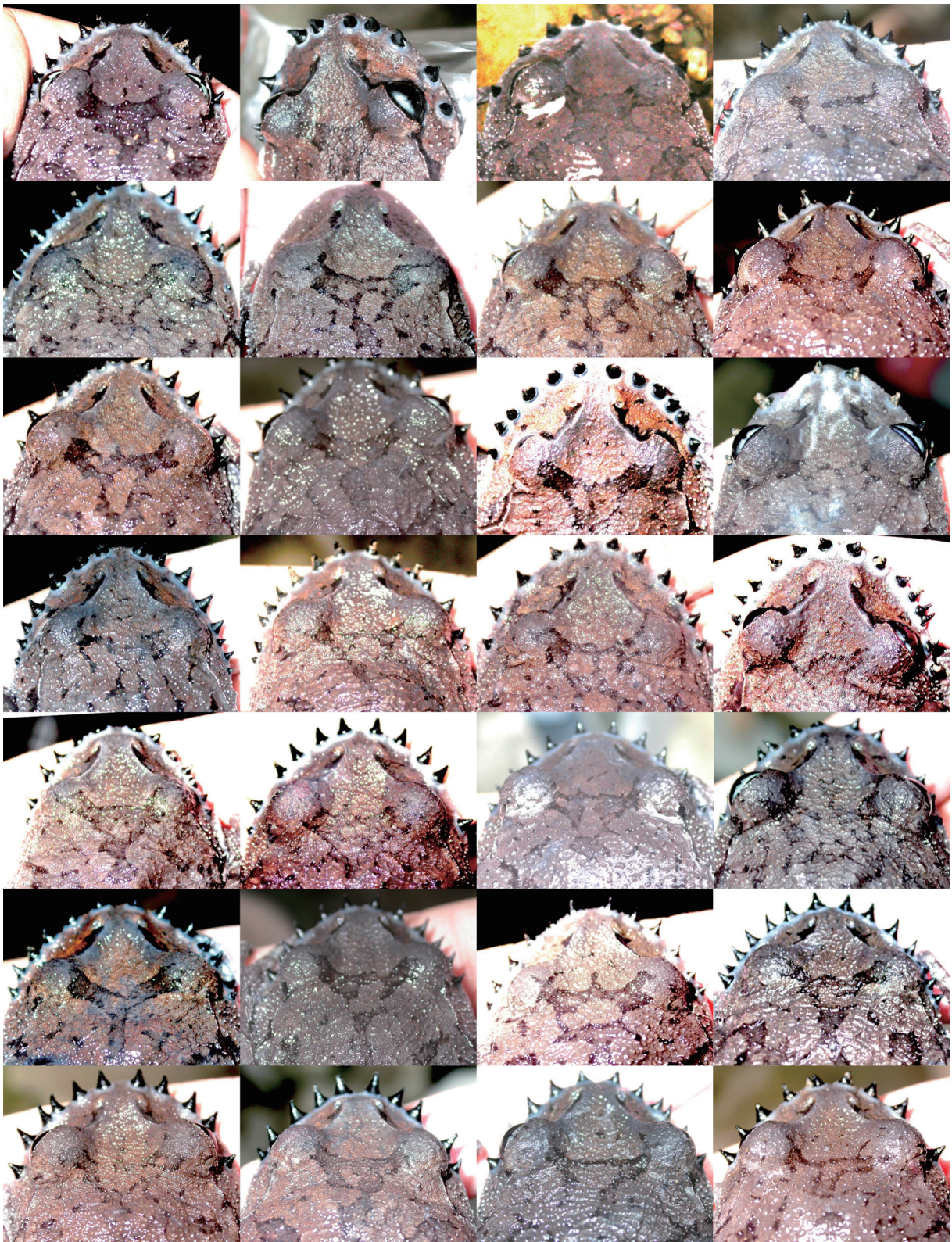
Figure 2 Individual-specific markings on top of the head of all 28 individuals recorded in 2009 at Site Emei.

(Figure 3). At both field sites, in a quiet environment, calls emitted from some submerged nests could be heard by us at a distance of up to 10 m. To the human ear, such a note sounded like "ah". Using parameters averaged over different calls of an individual, the following description was based on 208 advertisement calls from 27 males

Table 1 Parameters of advertisement calls of *Leptobrachium boringii* and *L. leishanense* recorded in the field. The number of males examined is given as n. Parameters of different calls of a male were averaged. For the single-note call, 10 calls were measured for each individual. For the two-note call, 3 to 10 calls with an average of 7 calls were measured for each individual. For the three-note call, 1 to 3 calls with an average of 2 calls were measured for each male. Values are means ± SD with ranges in parentheses.

Parameter	Site Emei (*L. boringii*)			Site Badagong (*L. boringii*)		Site Leigong (*L. leishanense*)	
	Single-note call (10–13°C, n = 10)	Two-note call (10–13°C, n = 10)	Three-note call (9–12°C, n = 6)	Single-note call (8°C, n = 2)	Two-note call (8°C, n = 1)	Single-note call (n = 1)	Two-note call (n = 1)
Call duration (s)	0.15 ± 0.02 (0.11–0.18)	1.01 ± 0.10 (0.90–1.16)	1.70 ± 0.15 (1.54–1.97)	0.22 (0.21–0.23)	1.26	0.20	1.28
Single-note call rate	0.065 ± 0.022 (0.035–0.099)			0.054 (0.042–0.065)		0.088	
Note 1 duration (s)	0.15 ± 0.02 (0.11–0.18)	0.17 ± 0.02 (0.14–0.19)	0.16 ± 0.02 (0.13–0.18)	0.22 (0.21–0.23)	0.23	0.20	0.21
Note 2 duration (s)		0.20 ± 0.02 (0.17–0.23)	0.18 ± 0.02 (0.15–0.19)		0.26		0.45
Note 3 duration (s)			0.20 ± 0.02 (0.16–0.23)				
Internote interval 1 (s)		0.64 ± 0.08 (0.55–0.78)	0.55 ± 0.06 (0.47–0.65)		0.77		0.62
Internote interval 2 (s)			0.60 ± 0.08 (0.53–0.74)				
Note repetition rate		1.25 ± 0.12 (1.08–1.41)	1.35 ± 0.13 (1.14–1.50)		1.00		1.22
Pulses in note 1	13.4 ± 1.6 (10.9–16.2)	12.7 ± 2.5 (9.4–17.3)	12.8 ± 3.9 (9.0–18.0)	17.3 (16.7–17.8)	18.0	19.9	21.3
Pulses in note 2		17.1 ± 3.2 (12.3–21.8)	15.8 ± 3.6 (12.5–20.3)		18.9		25.3
Pulses in note 3			18.1 ± 4.4 (12.5–24.0)				
Pulse rate of note 1	95 ± 8 (85–110)	80 ± 11 (65–100)	85 ± 20 (63–114)	79 (77–82)	79	99	103
Pulse rate of note 2		88 ± 14 (68–111)	93 ± 19 (72–117)		74		58
Pulse rate of note 3			91 ± 18 (74–116)				
Dominant frequency of note 1 (kHz)	0.9 ± 0.2 (0.7–1.1)	0.8 ± 0.2 (0.4–1.2)	0.9 ± 0.2 (0.7–1.1)	0.8 (0.6*–0.9)	0.6*	0.8	0.9
Dominant frequency of note 2 (kHz)		0.8 ± 0.2 (0.4–1.2)	0.9 ± 0.2 (0.7–1.2)		0.6*		0.7
Dominant frequency of note 3 (kHz)			0.9 ± 0.2 (0.7–1.1)				

*: Based on calls recorded at a distance of more than 1 m.

recorded in the field at 8–13°C from both sites. Details of calls with 1–3 notes are given in Table 1. Call duration ranged from 0.11 s (single-note) to 3.12 s (five-note). A note consisted of 9.0–24.0 pulses and lasted 0.11–0.26 s, with a pulse rate of 52–117/s. The dominant frequency of notes varied from 0.4 to 1.2 kHz, and usually spanned about 0.5 kHz. Weak harmonics at higher frequencies were also present. The fundamental frequency varied considerably within a note, and was found mostly in the range of 0.04–0.14 kHz. In a call with two or more notes, the internote interval lasted 0.47–0.78 s. These calls were not made by simply repeating the first note, but rather were composed of notes with increasing pulses and durations. In the two-note call, on average, the 2nd note
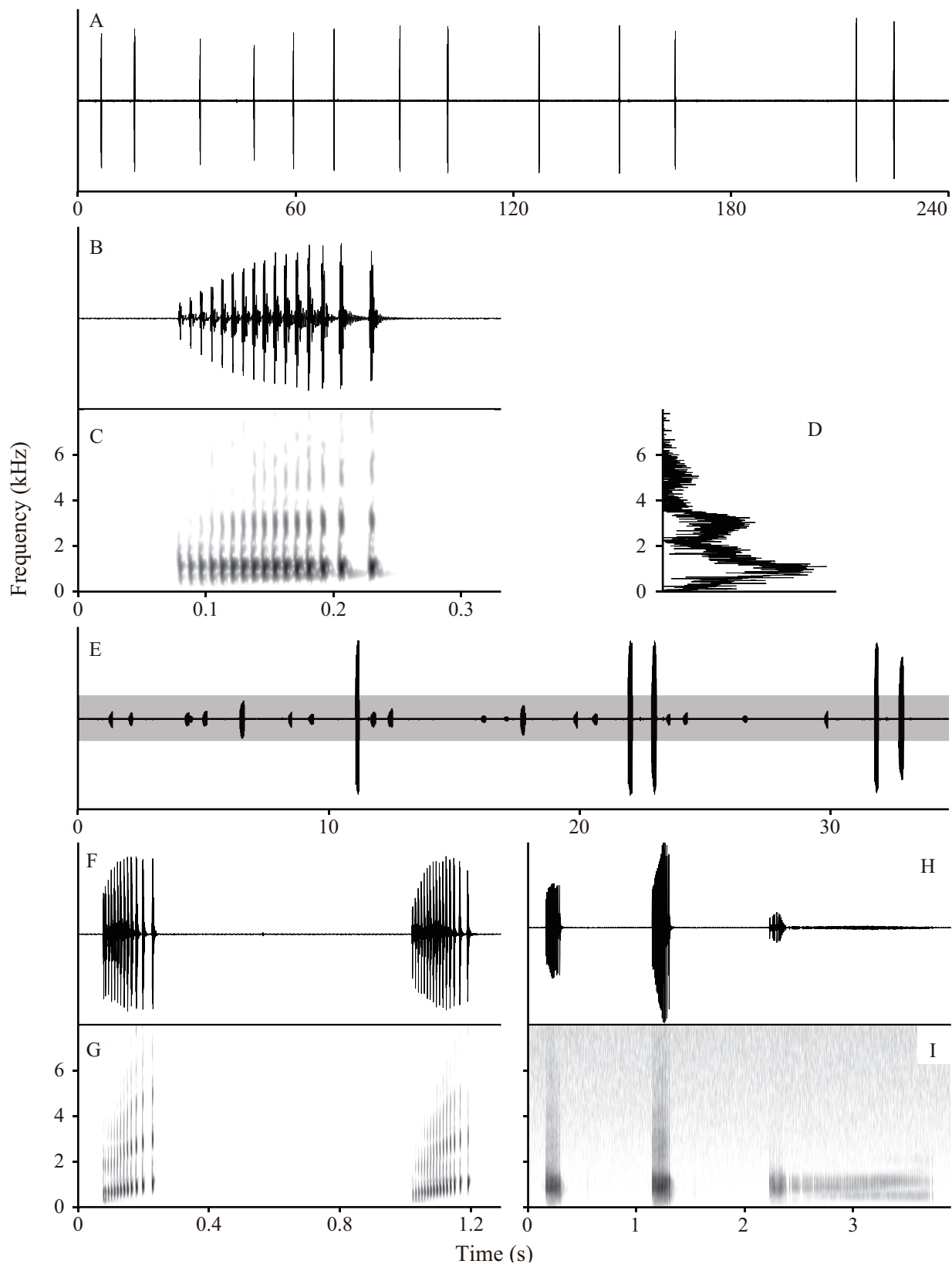
Figure 3 Calls of male *Leptobrachium boringii* from Site Emei. A: Oscillogram of ten advertisement calls of a male, recorded in the field at a water temperature of 11°C; B: oscillogram, C: sonogram, and D: power spectrum of a single-note call of the ten calls; E: oscillogram of three advertisement calls of a second individual, recorded in a natural chorus at a water temperature of 13°C. Calls in the shaded region were produced by other males. F: Oscillogram and G: sonogram of a two-note call of the three calls; H: oscillogram and I: sonogram of a courtship call of another individual in an artificial nest. All sonograms are broad-band (260 Hz) displays.

contained 4.4 (34%) more pulses and lasted 0.03 s (20%) longer than the 1st note (paired *t*-tests, n = 10, *P* < 0.001 for both tests). In the three-note call, the 2nd and 3rd notes contained 3.0 (23%) and 2.3 (14%) more pulses and lasted 0.02 s (12%) and 0.03 s (14%) longer than the 1st and 2nd notes, respectively (paired *t*-tests, n = 6, *P* < 0.013 for all tests). These differences were statistically significant at the 5% level after sequential Bonferroni correction. In the four-note call recorded at 11°C from Mt. Emei, the 1st through 4th notes consisted of 9 (0.18 s), 10 (0.18 s), 14 (0.24 s), and 17 (0.23 s) pulses, respectively, and were repeated at a rate of 1.29/s. In the five-note call recorded at 11°C from the same site, the 1st through 5th notes were composed of 9 (0.15 s), 10 (0.17 s), 13 (0.22 s), 15 (0.23 s), and 15 (0.23 s) pulses, respectively, with a note repetition rate of 1.39/s.

The courtship call was likely produced by adding a prolonged, trill-like last note of low intensity to an advertisement call (Figure 3). The parameters for the portion of the call before the last note were similar to those for the advertisement call (Tables 1 and 2). The last note contained about 105–142 pulses, which were often grouped as several clusters and repeated at generally increasing intervals. In 12 of the 13 calls analyzed, the intensity of approximately the first 1/8 duration of the last note was much higher with respect to that of the rest. However, the dominant frequencies of both portions were largely similar. The dominant frequency measured over the entire last note, 0.7–1.0 kHz with an approximately 0.5 kHz span, was similar to that of the other note(s). Frequency modulation and weak harmonics at frequencies higher than the dominant frequency were also observed in the last note.

The male might have increased the call rate when a female was present, which had been observed in other frogs (Wells, 1988; Gerhardt and Huber, 2002). During the night of 2 March 2006, when male #0014 was alone in the artificial nest, eight single-note advertisement calls were repeated at a rate of 0.025/s. After a female was introduced into the nest, 21 multi-note advertisement and courtship calls were delivered at a rate of 0.103/s. The internote intervals (0.69–1.10 s) of these multi-note calls appeared to be slightly longer than those (0.47–0.78 s) of calls recorded in the field (Tables 1 and 2), which might be explained by the possible temperature difference between the artificial and natural nests (e.g., McLister, 2001) and/ or by the fact that the former were recorded while the frogs were moving around in the nest (see below).

3.2 Male calls of *L. leishanense* Two types of calls were recorded from submerged nests. One male calling alone in the field produced a two-note advertisement call after usually several single-note calls (Figure 4). In the artificial nest, single-note and two-note calls similar to the advertisement calls were recorded, as well as courtship call-like vocalizations, including calls with two or three notes. As two males were kept in this nest with females, only one representative call of each kind of calls was analyzed. No individuals were observed giving calls out of the water.

The note of the advertisement and advertisement-like calls was composed of many clear pulses. For such a note, the trends of the change in pulse rate, change in intensity, and frequency modulation were similar to those of *L. boringii* (Figures 3 and 4). In a quiet environment, the calls of *L. leishanense* were audible and also sounded like "ah". Parameters based on 10 single-note and 7 two-note advertisement calls of a male and 1 single-note and 1 two-note advertisement-like calls were listed in Tables 1 and 2. Among them, the single-note call rate, 1st note duration, pulse number and pulse rate of the 1st note, internote interval, and dominant frequency of notes were similar to those obtained in *L. boringii*. An approximately 0.5 kHz span of the dominant frequency and weak harmonics at higher frequencies were also observed. The fundamental frequency varied over the note duration, and generally was in the range of 0.05–0.12 kHz. In calls with two notes, the last note contained more pulses and lasted longer than the 1st note, which was consistent with the trend found in *L. boringii*. However, with longer duration and lower pulse rate, the last note of *L. leishanense* was readily distinguished from that of the multi-note calls of *L. boringii*.

Similar to the courtship call of *L. boringii*, the courtship-like call of *L. leishanense* was likely produced by adding a trill-like last note of low intensity and long duration to an advertisement call (Figure 4; Tables 1 and 2). At both the beginning and ending of the last note, pulses were delivered at higher intensities. Between them, single or two closely spaced pulses at lower intensities were delivered at a roughly constant rate. The dominant frequency of the last note ranged from 0.2 to 0.5 kHz, with a span of approximately 0.2 kHz. Weak harmonics at frequencies higher than the dominant frequency were detected.

3.3 Behavioral observations of *L. boringii* On the night of 2 March 2006, male #0014 raised its body from the bottom of the artificial nest and switched from single-note advertisement calls to multi-note advertisement and courtship calls in response to an approaching female. When producing these multi-note calls, it lifted the head

Table 2 Comparison between different types of calls. The number of calls examined is given as n. AD = advertisement (*Leptobrachium boringii*) or advertisement-like (*L. leishanense*) calls; CT = courtship (*L. boringii*) or courtship-like (*L. leishanense*) calls; DF = dominate frequency. Values are means ± SD or means with ranges in parentheses.

Parameter	Site Emei (*L. boringii*) male #0014[a]			Site Emei (*L. boringii*) male #1001[b]	Site Leigong (*L. leishanense*)[c]	
	1-note AD (n = 8)/ 2-note CT (n = 6)	2-note AD (n = 8)/ 3-note CT (n = 4)	3-note AD (n = 2)/ 4-note CT (n = 1)	1-note AD (n = 11)/ 2-note CT (n = 2)	1-note AD (n = 1)/ 2-note CT (n = 1)	2-note AD (n = 1)/ 3-note CT (n = 1)
Call duration (s)	0.14 ± 0.02/ 2.32 ± 0.17	1.44 ± 0.35/ 3.45 (3.15–3.99)	2.16 (2.11–2.20)/ 4.08	0.16 ± 0.01/ 1.93 (1.87–1.99)	0.20/ 4.77	1.41/ 3.74
Note 1 duration (s)	0.14 ± 0.02/ 0.17 ± 0.02	0.16 ± 0.01/ 0.17 (0.14–0.20)	0.17 (0.16–0.18)/ 0.16	0.16 ± 0.01/ 0.16 (0.16–0.17)	0.20/ 0.23	0.23/ 0.23
Note 2 duration (s)		0.18 ± 0.02/ 0.18 (0.17–0.22)	0.17 (0.16–0.17)/ 0.18			0.59/ 0.53
Note 3 duration (s)			0.21 (0.21–0.21)/ 0.21			
CT last note duration (s)	1.36 ± 0.16	1.39 (1.33–1.48)	1.32	1.20 (1.16–1.24)	3.96	1.90
Internote interval 1 (s)		1.10 ± 0.35/ 0.89 (0.70–1.32)	0.72 (0.69–0.75)/ 0.69			0.59/ 0.56
Internote interval 2 (s)			0.88 (0.79–0.97)/ 0.78			
CT last internote interval (s)	0.79 ± 0.06	0.82 (0.68–0.95)	0.73	0.57 (0.56–0.58)	0.58	0.53
Pulses in note 1	12.4 ± 1.3/ 18.8 ± 4.1	15.1 ± 3.2/ 16.5 (14–20)	14 (13–15)/ 16	13.5 ± 0.8/ 14.5 (13–16)	17/ 18	19/ 23
Pulses in note 2		19.1 ± 6.4/ 19.5 (16–27)	15 (15–15)/ 17			28/ 30
Pulses in note 3			17.5 (17–18)/ 20			
Pulses in CT last note (approx.)	142 ± 26	134 (118–153)	105	106 (104–107)	89	36
Pulse rate of note 1	90 ± 5/ 117 ± 31	101 ± 24/ 100 (94–113)	87 (85–89)/ 109	91 ± 6/ 101 (95–107)	87/ 90	86/ 104
Pulse rate of note 2		110 ± 22/ 111 (102–125)	92 (92–93)/ 96			49/ 58
Pulse rate of note 3			84 (83–85)/ 91			
DF of note 1 (kHz)	1.1 ± 0.1/ 0.9 ± 0.1	1.0 ± 0.1/ 0.9 (0.8–0.9)	0.9 (0.9–1.0)/ 1.0	0.8 ± 0.0/ 0.8 (0.8–0.8)	0.5/ 0.7	0.5/ 0.5
DF of note 2 (kHz)		0.9 ± 0.1/ 0.9 (0.8–1.1)	1.0 (0.9–1.0)/ 0.9			0.5/ 0.5
DF of note 3 (kHz)			1.0 (1.0–1.0)/ 1.0			
DF of CT last note (kHz)	0.8 ± 0.2	0.8 (0.5–1.0)	1.0	0.7 (0.7–0.8)	0.5	0.2

[a]: The single-note advertisement calls were recorded when this male was alone in an artificial nest. The other calls of this male were recorded in the same night when a female was present.
[b]: The calls of this individual, including four two-note advertisement calls with a mean internote interval of 0.64 (0.57–0.78) s, were recorded in the field when a female was present, at a water temperature of 12°C.
[c]: Calls were recorded from an artificial nest with two males and two females.

and anterior trunk as well as lowered the posterior trunk and thighs. The female approached the male from the front, put her head beneath his, and pushed upward, which initiated physical contact. Subsequently, a series of pre-oviposition behaviors were observed. The male rapidly rubbed the top of the female's head left-and-right with his chin. The female wandered around in the nest and frequently lifted her body and touched the bottom surface of the roof rock. The male emitted calls and moved behind the female and achieved inguinal amplexus. The
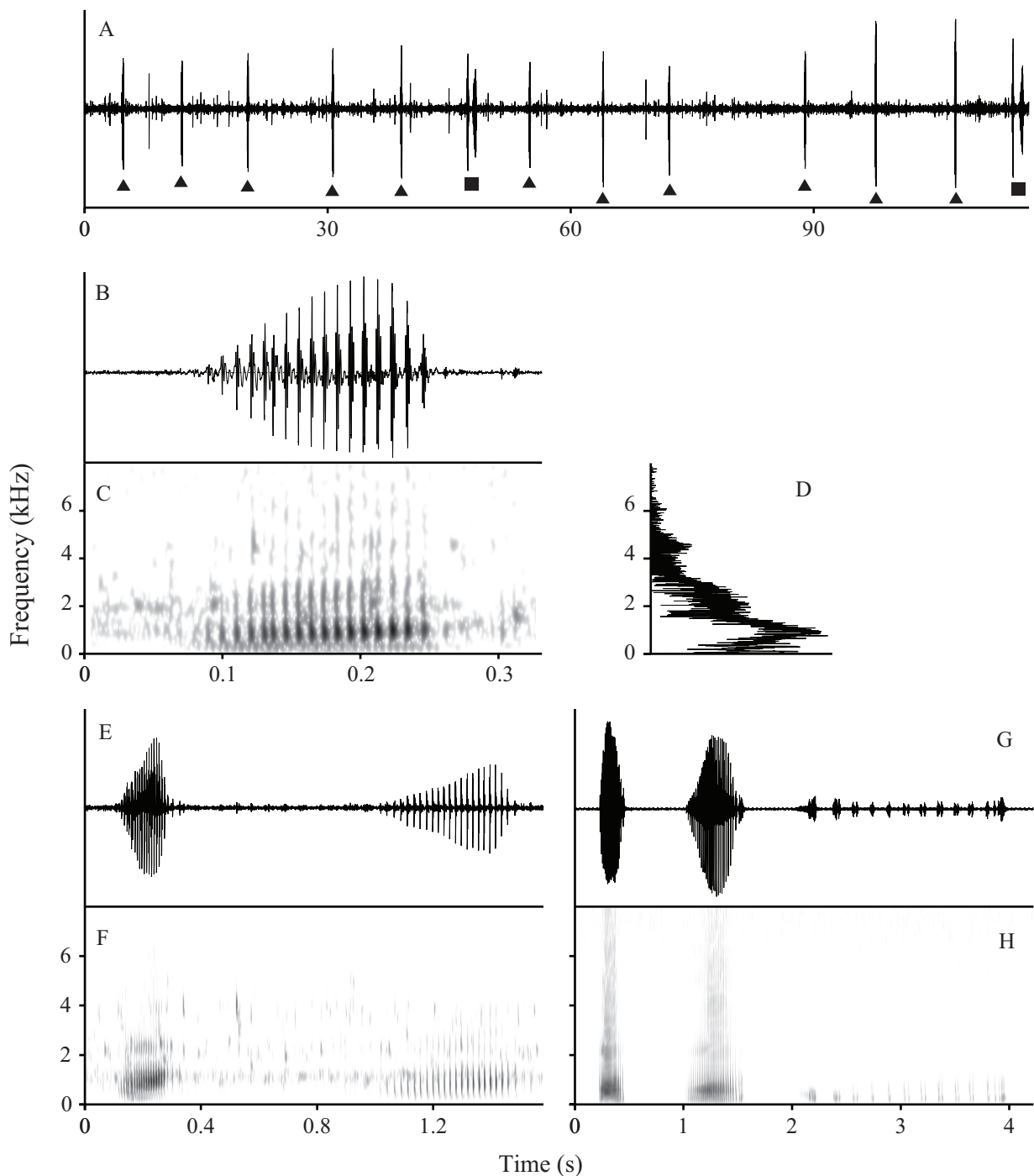
Figure 4 Calls of male *Leptobrachium leishanense* from Site Leigong. A: Oscillogram of 13 advertisement calls of a male in the field. Triangles indicate single-note calls and squares indicate two-note calls. B: Oscillogram, C: sonogram, and D: power spectrum of a single-note call of the 13 calls; E: oscillogram and F: sonogram of a two-note call of the 13 calls; G: oscillogram and H: sonogram of a supposed courtship call of a different male in an artificial nest. All sonograms are broad-band (260 Hz) displays.

amplectant pair continued to wander around, during which time the male and female kept on calling and touching the rock, respectively. The male released the female once the latter moved out from under the rock, but kept on emitting multi-note calls. The female swam up to extend her nostrils out of the water for a few minutes and then approached the male again. This sequence of events was repeated multiple times in the same night. On the next night, vocalizations of low intensity sounding like "coo" were produced during the oviposition process of the pair. During the two-night observation period, the male never left the nest.

Figure 5 Submissive posture of *Leptobrachium boringii* adopted by an intruder male (left) in the nest, which was occupied by a resident male (right). The egg mass was sired by the resident male.

During the night of 8 March 2006, another male (#1005) was placed in the aquarium when male #0014 was calling alone in the artificial nest with the egg mass. Throughout the night, male #1005 stayed outside the nest and emitted no calls. Compared with male #0014 (SVL 72.9 mm, weight 50 g on 9 March 2006), male #1005 had a similar body size (73.3 mm, 52 g on 5 March 2006). Three spines of male #1005 had fallen off before the observation, and male #0014 retained all of his spines. In the daytime of 9 March 2006, male #1005 entered the nest but behaved like a subdominant individual. It seldom moved, produced no calls, and usually stayed in the corners of the nest. When it remained motionless, it typically assumed a low, submissive posture, with the head and belly touching the substrate, and with the limbs being tightly bent toward its trunk (Figure 5). The resident male kept on calling and frequently approached the intruder, but without making aggressive physical contact. Whether specialized aggressive calls were produced during this process was not determined. On the night of the same day, after the resident male was removed, the intruder still remained silent, but moved freely in the nest and no longer assumed the submissive posture. No attempts were made by it to destroy the egg mass fathered by the resident male. Among five observations in the field of two males using a nest at different times, we did not observe any cases in which egg masses found with the first male were removed from the rock by the second male.

On the night of 27 February 2011, a case of male-male combat was recorded in the field. During the 50 minutes of observation, two males fought five rounds inside and outside the nest that each attempted to occupy alone. The fight started in the nest and could move on the streambed to a position approximately 1 m from the entrance of the nest. Spines were used as weapons in the fight. Each male usually lowered its head and struggled to put it under the body of the opponent, extended the forelimbs forward and slightly upward to try to grasp the opponent and steady it on the spines, and pushed forward with the hindlimbs. Attacks on the dorsal part of body were also observed. When a male successfully seized a lower position with its head beneath the opponent's body, the latter sometimes lifted the hindlimbs and posterior part of the body and attacked the dorsal side of the former with the spines. In doing this, the latter male extended the forelimbs to grasp and steady the former. During the fight, vocalizations of low intensity were heard, but not recorded. In the intervals between fights, a male was observed three times to move up and keep its nostrils out of the water for a short period of time. In one case, rising gas bubbles were observed as the male dived back down, thus suggesting it moved to the water surface to breathe.

The fight could have caused wounds such as red spot-like hemorrhages in the skin (Hudson *et al.*, 2011). Such red spots on the ventral side of the animal were
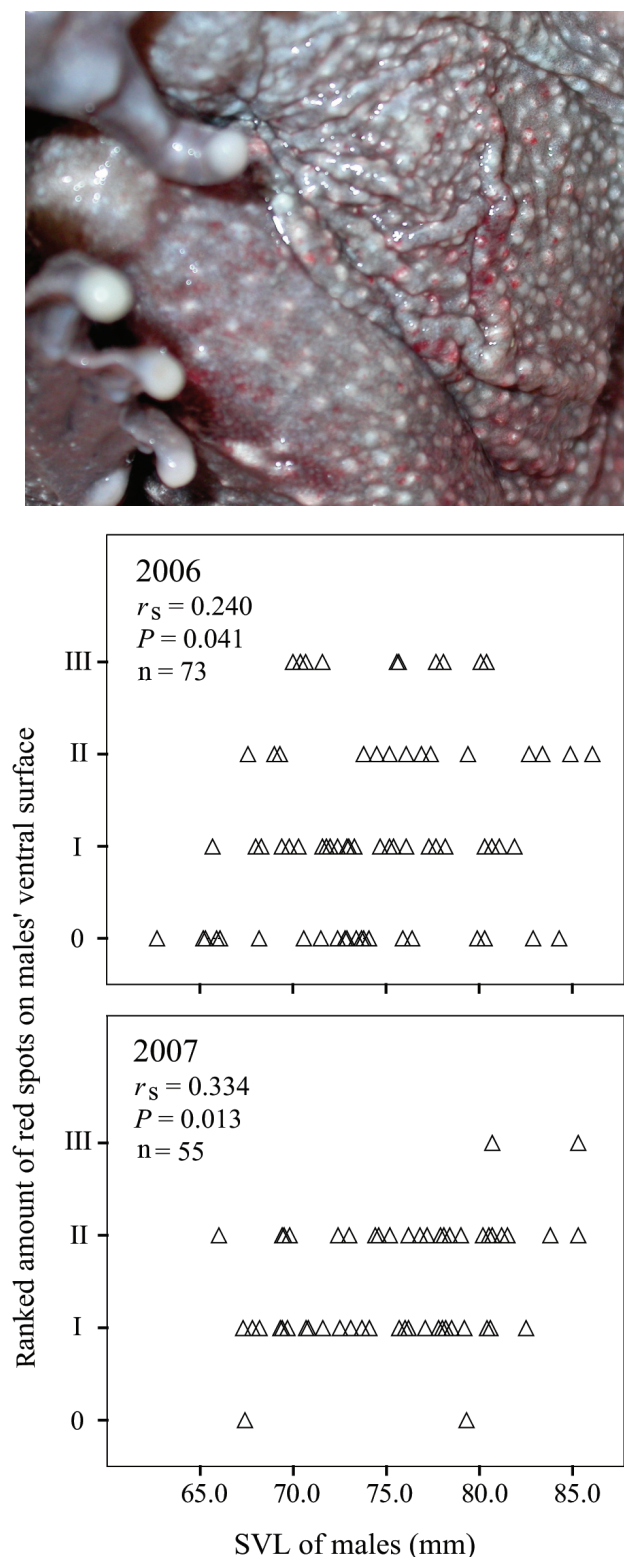
Figure 6　Correlation of male body size (SVL) with ranked amount of red spots on males' ventral sides (the photo). r_S = Spearman correlation coefficient. Both *P* values are two-tailed. The correlation was significant at 5% level after sequential Bonferroni correction. Data were collected from Site Emei.

recorded for individual frogs at Site Emei in 2006 and 2007, but were not interpreted as wounds until male-male combat was observed in 2011. Wounds could be readily distinguished, because the ventral side of the frog was lightly pigmented (Figure 6). With rare exceptions, similar densities of spots were recorded in different checks of the same frog. According to the densities, individuals were graded into four categories: 0 (no spots), I (a few spots), II (moderate amounts of spots), and III (many spots). A total of 73 males were ranked in 2006, including 52 (71%) Category I–III individuals. In 2007, 55 males were ranked, including 53 (96%) Category I–III individuals. No spots were found on females (n = 5). In both years, a significant positive correlation was detected between male body size (SVL) and the ranked number of spots (2006, Spearman correlation coefficient: r_S = 0.240, n = 73, *P* = 0.041; 2007, r_S = 0.334, n = 55, *P* = 0.013) (Figure 6). In 2004 at the same field site, a total of 28 males were found, but red spots were not noted, probably due to the much lower density of males (Howard, 1978; Zheng *et al.*, 2010). In 2007, red spots also were observed on the ventral side of males (n = 4) in a valley about 3 km from Site Emei. Three dead males were found on the streambed of Site Emei, two in 2006 and one in 2009. It was not likely that they were killed by predators, as their bodies were either complete or fairly complete. Two bodies were half rotten. The other one was found with one damaged eye, a hole of about 1 mm in diameter through the posterior back skin, and another such hole on the belly. Whether or not male-male combat caused these damages was not clear, but our observations suggested that a fight might have led to the death of males.

3.4 Spatial and temporal aspects of males at Site Emei
In 2006, two cases of smaller males closely associated with a larger male were recorded. Male SVL records of this breeding season ranged from 60.3 to 86.1 mm, with a mean of 74.2 mm and a SD of 5.4 mm (n = 81). In the first case, four smaller individuals were observed around two large individuals. In the daytime of 28 February, a large male and a small male were found at the same time in nest #31. On the bottom surface of the roof rock (about 70 cm × 40 cm), five egg masses were located at the central region of the nest and one egg mass occurred about 20 cm from them. When the rock was turned over, the large male (#0025, SVL 81.9 mm) was observed sitting under the five egg masses and the small male (71.9 mm) was found under the single egg mass. In a nest (#29, roof rock size about 30 cm × 30 cm) approximately 25 cm from nest #31, the other large male (80.1 mm) was found with five egg masses. Around these individuals
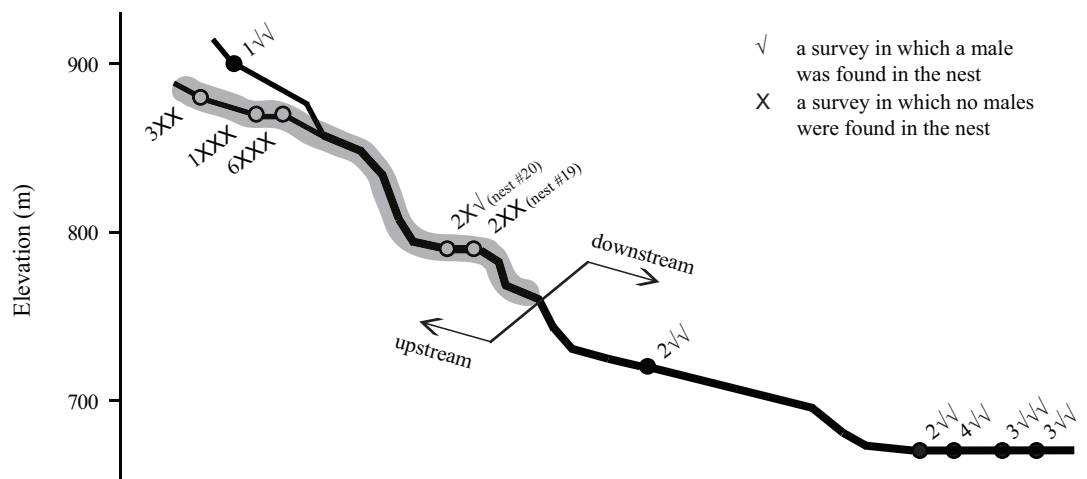
Figure 7 Distribution pattern of nests found with egg masses but no males at Site Emei in the 2009 early breeding season. All surveys were conducted between 17 and 21 February with an average interval of 40 hours. Each male was found alone in only one nest. Circles are nests. The number beside a nest indicates the number of egg masses in the nest. From the stream section in the shaded region, only two males were recorded (see text).

and at least 1.5 m away from any other nest, three small males were found on 1 March: male #0052 (74.4 mm, located in the daytime) was 50 cm from nest #29; male #1020 (77.3 mm, night) was 30 cm from nest #31; and a male (71.8 mm, night) was under the edge of the roof rock of nest #31. Covered by rocks of quite small sizes, the hiding places of males #0052 and #1020 were very narrow and no egg masses were ever found in such small places. In both rounds of follow-up checks conducted on 15 March and 27 March, the largest male (#0025) stayed in nest #31, a new male stayed in nest #29, all the other males had moved away. As the second case, in the daytime of 6 March, a large male (86.1 mm) and a female were found with two smaller males (73.4 and 76.4 mm) in one nest. Five egg masses were counted on the bottom surface of the roof rock (about 80 cm × 55 cm), four in the central region of the nest and one approximately 20 cm from them. The single egg mass looked very fresh and was probably laid by this female, because no eggs were visible through its semi-transparent abdominal wall. In the follow-up check carried out on 16 March, only the largest male was observed in the nest. In both cases, the position of the single egg mass was close to the edge of the roof rock.

Early in the 2009 breeding season, some nests were observed with egg masses, but without a male. A total of 12 nests were found with egg masses, and 11 of them were checked two or three times with an average interval of 40 hours. All four unoccupied nests with egg masses were from the upper half of the stream (Figure 7). A case of a male present only in the last check also from the

upper part of the stream (nest #20). Males that mated in unoccupied nests were not likely to have moved to other places in the breeding stream. In addition to the case of nest #20, another male was found in the stream section holding all the unoccupied nests. In the last round of checks, a male was located under a rock 45 cm from nest #19 (Figure 7). Within each of the occupied nests, a single male was continuously observed. Regardless of how nest #20 was grouped, the distribution pattern of unoccupied nests was significantly different from random (likelihood ratio chi-square test, n = 11, P = 0.002 or 0.009). During the middle and late parts of the breeding seasons in 2004, 2006, and 2007, among the 26, 35, and 41 nests that contained egg masses, only 2, 0, and 1 were found to be unattended, respectively. In 2009, a nest containing egg masses, but which was excluded from the analysis, occurred in the lower half of the stream. In the only check made in the daytime of 21 February, two males were found in it, and another three males were found under a pair of submerged cotton pants, which covered the area immediately outside the nest entrance. Interestingly, about five egg masses were laid on the underside of the pants, including two still attached to them in a doughnut-shape. This observation indicated that perhaps *L. boringii* is not specialized to attach the eggs on rocks. They appeared to construct nests under rocks because the rocks provided the only suitable materials for roof nesting in their natural environment. In 2011, egg masses were only observed in the lower half of the stream during our field work.

Two cases of a single nest being sequentially occupied by gradually larger and/or heavier males were recorded in

the early 2009 breeding season. No egg masses were laid in either nest. In one nest, the first male was recorded on 17 February, this male (SVL 71.0 mm, weight 43.5 g) and the second male (73.1 mm, 45.5 g) were found at the same time on 18 February, and the third male (74.3 mm, 51 g) was found on 21 February. In the other nest, the first (65.5 mm, 35 g), second (63.1 mm, 38.5 g), and third (69.6 mm, 41.5 g) males were recorded on 17, 18, and 21 February, respectively. During the six days of field work in 2009, these were the only nests occupied by different males at different times. As the intervals between checks were relatively short, interactions might have occurred between these individuals. If this was true, then the observation suggested the importance of body size in interactions between male *L. boringii*.

4. Discussion

4.1 Underwater calls In the genus *Leptobrachium*, four species are capable of calling underwater, *L. boringii*, *L. leishanense*, *L. ailaonicum*, and *L. liui*, and these species are phylogenetically closely related (e.g., Brown *et al.*, 2009; Matsui *et al.*, 2010; Frost, 2011). Our observations and calls of *L. boringii* and *L. leishanense* confirm that both species typically call while completely submerged, as noted in a previous study (Wu and Yang, 1981). In northern Vietnam, male *L. ailaonicum* (as *L. echinatum*) can be located by their deep single-note calls in submerged nests (Dubois and Ohler, 1998), and the same occurs at Mt. Wuliang, southern China (D. Rao, 2009, unpublished data). A video of a male calling underwater in an artificial nest is available at http://big5.cctv.com/gate/big5/space.tv.cctv.com/video/VIDE1249924405106881. Male *L. liui* often are observed calling while partially submerged at the edge of nests. Similar to the observation in the frog *Lithobates palustris* (Given, 2008), when a male is disturbed by knocking the roof rock, it withdraws into the nest, yet continues calling underwater, thus causing its calls to sound deeper to the human ear (Gu *et al.*, 1986). Underwater calling is poorly documented in anurans. It is recorded in the Pipidae, Ceratophryidae, and Ranidae, and it possibly occurs in the Bombinatoridae (Given, 2005; Wells, 2007; Frost, 2011). Taking the phylogenetic position of *Leptobrachium* into consideration, the above findings verify that underwater vocalization also occurs in another major evolutionary lineage of anurans, the Pelobatoidea (Frost *et al.*, 2006). This supports the view that underwater acoustic communication may be more broadly distributed in anurans than previously realized (Given, 2005).

The advertisement calls of *L. boringii* and *L. leishanense* generally resemble the terrestrial calls of their congeners. Many species of *Leptobrachium* are reported to have terrestrial advertisement calls (e.g., Lathrop *et al.*, 1998; Rao *et al.*, 2006; Stuart *et al.*, 2006). Among them, detailed call data are available for ten species: *L. pullum*, *L. leucops*, *L. smithi*, *L. hasseltii*, *L. lumadorum*, *L. montanum* (for a lineage regarded as a distinct species in Matsui *et al.*, 2010), *L. abbotti*, *L. gunungense*, *L. mangyanorum*, and *L. tagbanorum* (Matsui *et al.*, 1999; Malkmus *et al.*, 2002; Brown *et al.*, 2009; Stuart *et al.*, 2011). The dominant frequency (0.5–2.0 kHz), note duration (0.08–0.44 s), and number of pulses in a note (10–40 for species for which data are available) of these terrestrial callers overlap extensively with those (0.4–1.2 kHz, 0.11–0.45 s, and 9–25) of the two species that call underwater (Table 1). In the terrestrial callers *L. pullum* and *L. leucops*, which are most closely related to *L. boringii* and *L. leishanense* (Brown *et al.*, 2009; Matsui *et al.*, 2010; Stuart *et al.*, 2011), the pulse rate (72–129/s) is similar to that of the latter two species (52–117/s). To the human ear, the calls of these four species also sound similar: "ah" in *L. boringii* and *L. leishanense*, "waaaaah" in *L. pullum*, and "wah" in *L. leucops* (Stuart *et al.*, 2011). Such similarity implies that the sound-producing mechanism of *L. boringii* and *L. leishanense* is probably not essentially different from that of the congeneric terrestrial callers.

4.2 Male-male combat The keratinized spines of *L. boringii* are employed as weapons in male-male combat, and this function of the spines is not unique to this species. In the aforementioned video of *L. ailaonicum*, male-male fighting using spines (butting) underwater and male butting a large conspecific or congeneric tadpole with spines are presented. As a weapon used in male-male combat, rhinoceros-like horns appear independently in males belonging to a diversity of animal groups, such as the rhinoceros beetle, *Trypoxylus dichotomus*, and mountain chameleon, *Chamaeleo montium* (Emlen, 2008; Alcock, 2009). For extant amphibians, such structures— strong keratinized spines on the head—are reported only in *Leptobrachium*. The discovery of their function makes *L. boringii* and *L. ailaonicum* a noteworthy case(s) of convergent evolution in male weaponry (Emlen, 2008; Alcock, 2009). Male *Leptobrachium* having strong maxillary spines are also unusual in having conspicuously enlarged arms during the breeding season (e.g., Wu and Yang, 1981; Ho *et al.*, 1999; Rao *et al.*, 2006; Stuart *et al.*, 2011). The seasonal enlargement of forelimbs in *L. boringii* may be associated with male combat.

Hypertrophied forelimbs of males occur in many anurans, often associated with male combat (Wells, 2007). Male *L. boringii* hold their opponents on their spines with the forelimbs. In this case, it seems likely that the increased arm strength will enhance the power of the weapon.

A weak but significant positive correlation between body size and the amount of wounds on the ventral body surface occurs among male *L. boringii* (Figure 6) for the field work seasons of 2006 and 2007. Although males also attack other body parts of opponents with spines (Hudson *et al.*, 2011), such ventral wounds occur in most (71%, 2006) or nearly all (96%, 2007) individuals, suggesting that the ventral side is frequently targeted in fights. As a plausible explanation for this trend, larger males are more likely to engage in combat and therefore experience more fighting and wounding. In many anuran species, larger males more often win intrasexual physical contests (e.g., Howard, 1978; Arak, 1983; Wagner, 1989). This phenomenon of larger males behaving more aggressively in male-male interactions is reported to occur in various animals (e.g., Hayashi, 1985; Keenleyside *et al.*, 1985; Townsend and Jaeger, 1998).

4.3 Nest inspection In the artificial nest of *L. boringii*, the female wanders around and frequently touches the bottom surface of the roof rock with her back. As indicated by the lack of final oviposition in the same night, this behavior, relying on tactile sense, might function as an inspection of a potential nest under very low illumination. In addition, the nest is covered by a rock and the peak breeding activity usually occurs at night (Wu and Yang, 1981; Zheng *et al.*, 2010). Through touching the nest roof, a female can detect attached egg masses. At Site Emei, in many cases two or more (up to ten) egg masses can be found within a nest. They usually are attached to the rock adjacent to each other and commonly in contact. Overlapping is minimal, when it occurs (Figure 1) (Zheng *et al.*, 2010). This non-random pattern may facilitate effective paternal care (Zheng *et al.*, 2010) as well as precluding egg masses from dropping off from the rock. Dropped eggs are likely to be washed away from the nest (Y. Zheng, personal observation), and the breeding stream is rich with potential predators of the developing embryos (Zheng *et al.*, 2010). Because movement of the pair is driven by the female during oviposition, the female is likely capable of determining the oviposition location and hence the egg mass location on the basis of her inspection of the nest (Zheng and Fu, 2007). Nest inspections by females before oviposition occur in other frogs (Kluge, 1981; Martins and Haddad, 1988).

4.4 Possible alternative male reproductive tactics Satellite male mating tactic may exist at Site Emei and four pieces of evidence lead us to propose this hypothesis. First, there are two recorded cases of smaller males gathering around a large male. Among these, only the large males are encountered with the egg masses in the follow-up checks. This meets the distribution pattern and body size differences among dominant and satellite males commonly observed in frogs with resource defense mating systems (Gerhardt and Huber, 2002; Wells, 2007). Further, in both cases, one egg mass occurs near the edge of the roof rock and a considerable distance away from the other egg masses. As discussed above, this phenomenon is unusual, but can be explained by successful female interception by satellite males (Gerhardt and Huber, 2002). Second, parentage analysis using microsatellite DNA markers reveals that some egg masses were sired by multiple males (Zheng *et al.*, 2010), and this can be explained by satellite males having fertilized some eggs either during or even after oviposition (Vieites *et al.*, 2004). Third, in the artificial nest, the resident male tolerates the presence of the intruder, which typically assumes a low, submissive posture (Figure 5). The posture may be adopted by satellite males to enter the dominant male's nest to avoid being attacked (Wells, 1977; Toledo and Haddad, 2005). Lastly, the courtship call of males differs from the advertisement call only by an additional last note of low intensity (Figure 3; Tables 1 and 2). The low intensity, and hence short-range, of the note could reduce the likelihood that other males, such as satellite individuals, will note the presence of a female and interfere with courtship (Given, 1993; Wells and Schwartz, 2007). To evaluate this hypothesis, precise call recording and parentage analysis are necessary.

Early in the 2009 breeding season, most nests containing egg masses in the upper stream did not have a male present yet egg masses in the downstream nests were found with males (Figure 7). For many amphibians, the initiation of breeding has been shown to be closely tied to rising temperatures (Wells, 2007), and high-altitude populations breeding later than lowland ones has been commonly reported in anurans (e.g., Pettus and Angleton, 1967; Berven, 1982; Beattie, 1985). In the early 2011 spring breeding season, when many egg masses were observed in the lower half of the stream, no egg masses were found in the upper stream. Egg masses were always or nearly always found with males in the middle and late parts of the breeding seasons in 2004, 2006, and 2007. These observations suggested that during 2009 some mated males had left the breeding stream in the very early

period of the breeding season.

A conditional reproductive tactic (Gerhardt and Huber, 2002) may be used by *L. boringii*. Mating and leaving early in a season, a male will lose opportunities of additional mating, but he will benefit from increased future mating success (Williams, 1966). The energetic costs of calling and male combat is high (e.g., Ryan, 1988; Wells, 2001), and a significant body mass reduction occurs in this population during the middle and late breeding season (Zheng *et al.*, 2010). Further, the male may also benefit from possible paternal care provided to his offspring by a subsequent male (Roldán and Soler, 2011). Several sources of data support this possibility. First, males fight each other for nests, and mating activity is concentrated in the early breeding season (Zheng *et al.*, 2010). When males are common, an empty nest is likely to be used by another male early in the season. If mating is successful, the male may stay in the nest for a longer period of time and provide paternal care, as is observed in the middle and late breeding season (Zheng *et al.*, 2010). Second, behavioral observations and follow-up checks of nests suggest that males do not attempt to remove egg masses fathered by other individuals. Parentage analyses reveal that all or some of the egg samples of some masses do not share alleles with the resident male, at least at one locus. Some of these egg masses appear to be sired by a non-resident male (Zheng *et al.*, 2010). This finding is based on egg sampling conducted late in the breeding season. The finding may also be explained by a prior resident male that mated and left early in the breeding season. Further research is required to test this explanation.

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